THE SUBSTRATE ANALOG BROMOPYRUVATE AS A SUBSTRATE, AN INHIBITOR AND AN ALKYLATING AGENT OF MALIC ENZYME OF PIGEON LIVER[1]

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Summary: Bromopyruvate is an alkylating agent of pigeon liver malic enzyme (malate dehydrogenase (decarboxylating), EC 1.1.1.40). It combines first with the enzyme to give an enzyme-bromopyruvate complex, then reacts with a proximal -SH group, resulting in the formation of a pyruvate derivative. Bromopyruvate is also a substrate for the reductase partial reaction, and a non-competitive inhibitor of L-malate in the overall oxidative decarboxylase reaction catalyzed by this enzyme. Modification of the -SH group by this compound is accompanied by concomitant loss of both oxidative decarboxylase activity and reductase activity on bromopyruvate. Inactivation of the overall activity is partially prevented by $NADP^+$ or NADPH, singly or in combination with L-malate.

Pigeon liver malic enzyme catalyzes two major reactions (Reaction 1 and 2) (1,2) and a minor reaction (Reaction 3) (3).

$$\text{L-malate} + NADP^+ \underset{}{\overset{Me^{2+}}{\rightleftharpoons}} CO_2 + \text{Pyruvate} + NADPH \qquad (1)$$

$$\text{Oxaloacetate} \xrightarrow{Me^{2+}} CO_2 + \text{Pyruvate} \qquad (2)$$

$$\text{Pyruvate} + NADPH \xrightarrow{Me^{2+}} \text{L-Lactate} + NADP^+ \qquad (3)$$

This enzyme is believed to contain a -SH group at or near the active center (4,5). Alteration of this group by either 5.5'-dithiobis-(2-nitrobenzoic acid) or N-ethylmaleimide results in the loss of oxidative decarboxylase activity (Reaction 1), accompanied by enhanced reductase activity on pyruvate (Reaction 3).

It will be shown in this communication that the substrate analog bromopyruvate alkylates the above -SH group, yielding a pyruvate derivative with altered catalytic activities. The kinetic parameters of bromopyruvate as a substrate, an inhibitor of L-malate, and an alkylating agent are also reported.

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Materials and Methods

Bromopyruvate was purchased from Calbiochem. Corp. It was dissolved in water and neutralized with either KOH or NaOH before each use. Other chemicals used in this study were obtained as described previously (6). Malic enzyme was purified and its oxidative decarboxylase activity assayed according to Hsu and Lardy (7). The reductase activity was assayed according to Tang and Hsu (5), using pyruvate as the substrate. Formation (or disappearance) of NADPH was monitored at 340 nm and 30°. Modification experiments were performed at either 0° or 24° by the addition of bromopyruvate to an enzyme solution at pH 7.5. The progress of the reaction was monitored by assaying for oxidative decarboxylase or reductase activity on small aliquots withdrawn at the designated time intervals.

Results and Discussion

Modulation of malic enzyme activities by bromopyruvate. Bromopyruvate, an analog of the substrate pyruvate reacts with malic enzyme to produce an alkylated derivative which is inactive as an oxidative decarboxylase. The time course of reaction is shown in Fig. 1. The pseudo-first-order plot for inactivation followed a linear course up to 65 minutes with 81% loss of activity. In other experiments under slightly different conditions, linearity was observed up to 95% inactivation. Inactivation of the overall activity by bromopyruvate is accompanied by parallel loss of the ability of this enzyme to catalyze the reduction of bromopyruvate (see page 4, and Figure 3 for details on this reaction), but not pyruvate. In contrast, the latter reaction was enhanced approximately 1.5 fold during the experimental period (Fig. 1). The rate of oxidative decarboxylase inactivation was highly temperature dependent. At a bromopyruvate concentration of 5 mM, the observed first-order rate constants (k_{obs}) were 0.025 and 0.23 $min.^{-1}$ at 0° and 24° respectively.

Dependence of the rate of inactivation on bromopyruvate concentration. Kinetics of oxidative decarboxylase activity loss was examined over a range of bromopyruvate

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The abbreviations used are: DTNB, 5.5'-dithiobis-(2-nitrobenzoic acid); NEM, N-ethylmaleimide; PMB, p-hydroxy mercuribenzoic acid; DTT, dithiothreitol; OAA, oxaloacetic acid.

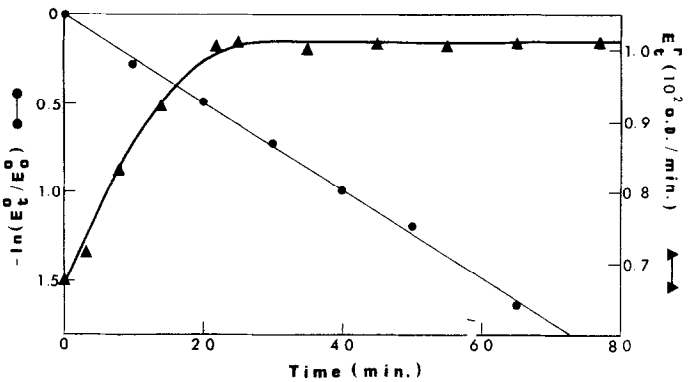

Fig. 1. The alteration of intrinsic activities of malic enzyme by bromopyruvate. The oxidative decarboxylase and reductase activities were examined in separate experiments under conditions described in "Materials and Methods." The reaction mixtures consisted of malic enzyme, 0.38 μM in 66.7 mM Tris-Cl buffer (pH 7.5); and bromopyruvate, 5 mM. Incubations were carried out at 0° in an ice-water bath. E^o, oxidative decarboxylase activity/0.99 μg enzyme; E^r, reductase activity/9.9 μg enzyme. Subscripts o and t denote activities at zero time and time t, respectively.

concentrations. The rate constants were calculated and plotted against bromopyruvate concentration (Fig. 2). The resultant hyperbolic curve resembles the Michaelis-Menten kinetic behavior of a substrate, rather than the linear plot expected of a simple second-order reaction. This saturation behavior is in accord with a two step reaction sequence whereby reversible binding of bromopyruvate occurs prior to alkylation as shown below:

$$E + \text{Br-pyr} \underset{}{\overset{K}{\rightleftharpoons}} E\text{--Br-pyr} \xrightarrow{k_2} E\text{-pyr} + Br^-$$

The reciprocal rate equation for the above reaction is $\frac{1}{k_{obs}} = \frac{K}{k_2 [I]} + \frac{1}{k_2}$ (8)

where k_{obs}, k_2, K, and [I] are respectively the observed first-order rate constant, the limiting first-order rate constant, the dissociation constant of the E---Br-pyr complex, and the bromopyruvate concentration. According to this equation, a plot of $1/k_{obs}$ against $1/[I]$ would be linear. Such a plot is shown in Fig. 2 insert. The values of k_2 and K calculated from this plot were 0.067 $min.^{-1}$ and 8.33 mM, respectively.

Effects of substrates on the kinetics of bromopyruvate inactivation. Table I shows the effects of reaction components on the rate of oxidative decarboxylase inactiva-

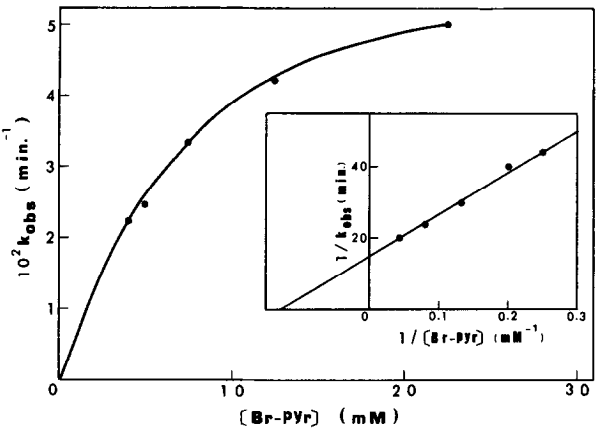

Fig. 2. Effect of bromopyruvate concentration on the rate of oxidative decarboxylase inactivation.
Conditions of incubation were as described in Fig. 1, except bromopyruvate concentration was varied as indicated. Pseudo-first-order plots at all bromopyruvate concentrations were linear; the inactivation rates (k_{obs}) were obtained from the slopes of these plots. Fig. 2 insert shows a linear double reciprocal plot of this data.

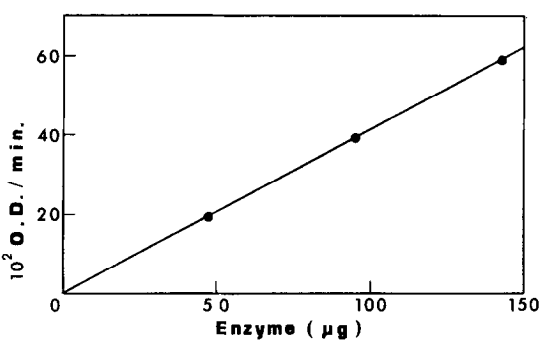

Fig. 3. Reduction of bromopyruvate as a function of enzyme concentration.
The reaction mixture contained Triethanolamine-Cl buffer (pH 7.4), 200 µmoles; $MgCl_2$, 24 µmoles; bromopyruvate, 3.75 µmoles; NADPH, 0.32 µmole; and enzyme in a volume of 3.0 ml.

tion. The rate constants are listed in the order of increasing magnitude. The strong protection by DTT clearly indicates that alkylation of -SH group is involved. The carboxylic acid substrates when added singly in the absence of the nucleotide cofactor either accelerated the inactivation or were ineffectual, comparable to their effects on the rate of modification by DTNB or NEM (4,5). On the other hand, protection was observed with either $NADP^+$ or NADPH. This protection was enhanced by L-malate, but decreased by either OAA or pyruvate. These results are compatible

Table I. Effects of reaction components on the rate of bromopyruvate inactivation.

Conditions in Experiment 1 were as described in Fig. 1, except a bromopyruvate concentration of 10 mM was used. Experiment 2 was carried out at 24° at a bromopyruvate concentration of 0.2 mM.

Additions	k_{obs} (min.$^{-1}$)	
	Experiment 1	Experiment 2
1. DTT (16.7 mM)	0.001	--
2. L-malate (1.16 mM) + $NADP^+$ (75.6 μM)	0.008	0.020
3. NADPH (18.3 μM)	0.013	--
4. $NADP^+$ (75.6 μM)	0.017	--
5. OAA (4.45 mM) + $NADP^+$ (75.6 μM)	0.028	--
6. None	0.030	0.044
7. Pyruvate (22.2 mM) + NADPH (18.3 μM)	0.030	--
8. Pyruvate (22.2 mM)	0.030	0.047
9. L-malate (1.16 mM)	0.030	0.053
10. $MnCl_2$ (6.67 mM)	0.033	--
11. OAA (4.45 mM)	0.043	--

with the ordered mechanism where the nucleotide cofactors combine with the free enzyme, resulting in a conformational change which alters (and strengthens) the binding of carboxylic acid substrates (9).

Bromopyruvate as a substrate of the reductase reaction, and an inhibitor of the oxidative decarboxylase reaction. The above results suggests that bromopyruvate acts as an active-site directed alkylating agent. Therefore, it is not surprising to find that this compound also functions as a substrate and an inhibitor of malic enzyme. Fig. 3 shows the correlation between the rate of bromopyruvate dependent NADPH oxidation and enzyme concentration. The effect of substrate concentration on reaction rate is shown in Fig. 4. For purposes of comparision, double reciprocal plots on pyruvate and bromopyruvate were obtained under otherwise identical conditior

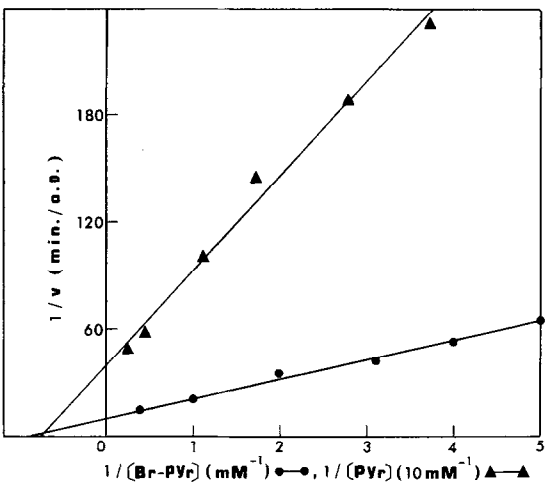

Fig. 4. Effect of bromopyruvate and pyruvate concentrations on the reductase reaction.
Conditions were the same as in Fig. 3, except that the experiments were carried at pH 7.5 in 50 mM Tris-Cl buffer using either pyruvate or bromopyruvate as the variable substrate. The amount of enzyme used in each assay was 47 µg.

It should be noted that bromopyruvate is a much better reductase substrate. The apparent Michaelis constant (k_m) of bromopyruvate (1.1 mM) is approximately one order of magnitude lower than that of pyruvate (13.3 mM), or the dissociation constant of the E---Br-pyr complex (K = 8.33 mM); moreover, the apparent maximal velocity (Vm) of the brominated derivative is four times higher (i.e. 0.048 versus a Vm value of 0.012 µmole of NADPH oxidized/min. for pyruvate).

Bromopyruvate was also found to function as a classical non-competitive inhibitor of L-malate in the oxidative decarboxylase reaction (Fig. 5). The slope (K_{is}) and intercept (K_{ii}) inhibition constants were identical, yielding a value of 0.8 mM. This value is similar in magnitude to its Michaelis constant of 1.1 mM in the reductase reaction, but much lower than the slope inhibition constant of pyruvate (14 mM at pH 7.0) obtained in early studies (9). Therefore, bromopyruvate is also a more potent inhibitor of oxidative decarboxylase than pyruvate.

Evidence on the site of alkylation by bromopyruvate. It has been shown (4,5) that reaction of DTNB or NEM with an -SH group at or near the active center of malic enzyme yields derivatives which are incapable of catalyzing the oxidative decarboxylase reaction, while possessing supra-active reductase activity on pyruvate. The

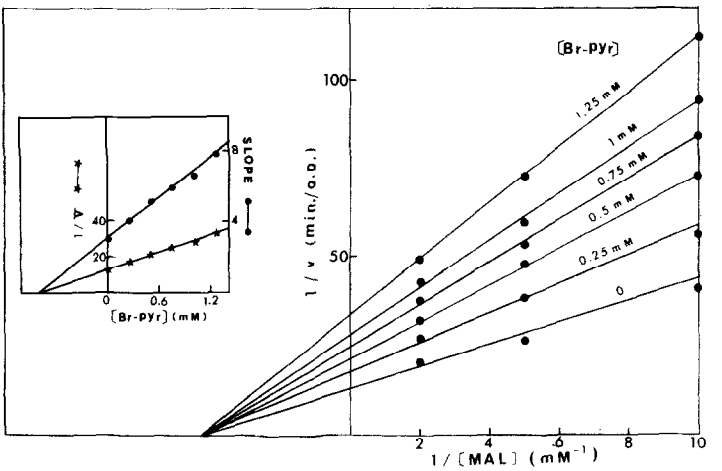

Fig. 5. Inhibition of oxidative decarboxylase activity by bromopyruvate. Oxidative decarboxylase assays were performed as described in "Materials and Methods," except the Triethenolamine-Cl buffer was adjusted to pH 7.5. The bromopyruvate and L-malate concentrations were varied as indicated. The amount of enzyme used in each assay was 2 µg. Fig. 5 insert shows plots of intercept and slope against bromopyruvate concentration.

qualitative similar effects of bromopyruvate suggests that the same -SH group may be involved. This suggestion is confirmed by results shown in Table II, where experimental evidence is presented to show that irreversible inactivation by bromopyruvate was prevented almost quantitatively by blocking this -SH group with DTNB. Similarly, we can conclude that PMB also reacts with this group, since it was nearly as effective in blocking the bromopyruvate reaction.

Results presented in this communication indicate that bromopyruvate acts as an affinity label which reversibly combines with malic enzyme, followed by reaction with the proximal -SH group to yield a pyruvate derivative of this enzyme. The availability of this derivative will be instrumental for an exploration on the geometry of the active center. Further kinetic experiments are underway to delineate the unique properties of bromopyruvate as an inhibitor, an alkylating agent, and a substrate for reduction. Kinetic analysis of substrate protection will yield additional information on the binding order and affinities of binary and ternary enzyme complexes. Moreover, mapping of the peptide segment containing the -SH group may be possible, pending on the outcome of experiments designed to demonstrate the stoichiometry of bromopyruvate reaction.

Table II. Protection of malic enzyme from bromopyruvate inactivation by DTNB or PMB.

Malic enzyme (25 µg) in 95 mM Tris-Cl buffer (pH 7.5) was incubated with DTNB (80 µM) or PMB (51 µM) at 24° until loss of oxidative decarboxylase activity was complete. Bromopyruvate (25 mM) was then added and allowed to react for 10 min.[1] The reaction mixtures were chilled to 0°[2], followed by the addition of DTT to 25 mM. Maximum recovery of oxidative decarboxylase activity by DTT was obtained after 10 min. (DTNB treatment) or 5 min. (PMB treatment). The control samples were treated with sulfhydryl reagents as above, except DTT was added to each vessel immediately before bromopyruvate, and allowed to regain oxidative decarboxylase activity at 0° for the time period specified above. For purposes of calculation, the recovery by control samples was taken as 100%.

Pretreatment	Recovery of activity by DTT (%)
DTNB	83
PMB	68

[1]These conditions were chosen to allow complete inactivation by bromopyruvate without pretreatment by DTNB or PMB.

[2]Chilling was necessary to minimize alkylation of the DTT regenerated enzyme by bromopyruvate.

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